

IN THE UNITED STATES PATENT AND TRADEMARK  
APPLICATION FOR LETTERS PATENT

TITLE: POLYNUCLEOTIDE VACCINE FORMULA IN PARTICULAR  
AGAINST BOVINE RESPIRATORY PATHOLOGY

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CLAIMS: 11 (1 independent)

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It therefore appears necessary to try to develop an effective prevention against the principal viruses involved in bovine respiratory pathology.

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Associations of vaccines against certain viruses responsible for bovine respiratory pathology have already been proposed in the past.

5 The associations developed so far were prepared from inactivated vaccines or live vaccines and, optionally, mixtures of such vaccines. Their development poses problems of compatibility between valencies and of stability. It is indeed necessary to ensure both the compatibility between the different vaccine valencies,  
10 whether from the point of view of the different antigens used or from the point of view of the formulations themselves, especially in the case where both inactivated vaccines and live vaccines are combined. The problem of the conservation of such combined vaccines  
15 and also of their safety especially in the presence of an adjuvant also exists. These vaccines are in general quite expensive.

Patent Applications WO-A-90 11092, WO-A-93 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the  
20 recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host's cells, the antigen inserted into the plasmid. All the routes of administration have been proposed (intraperitoneal,  
25 intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animal's skin (Tang et al., Nature 356,  
30 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992).

35 The polynucleotide vaccines may also use both naked DNAs and DNAs formulated, for example, inside cationic lipid liposomes.

G.J.M. COX has already proposed polynucleotide vaccination against type 1 bovine herpes virus in J. of

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Virology, Volume 67, No. 9, September 1993, 5664-5667. The authors have also described plasmids integrating the gI (gB), gIII (gC) and gIV (gD) genes.

5 In Vaccine, Volume 13, No. 4, 415-421, 1995, J.E. CROWE presents a general review of the different methods of vaccination against respiratory syncytial virus and against type 3 parainfluenza virus. This review reexamines all the possibilities offered by the current vaccination techniques and simply suggests that  
10 the polynucleotide immunization technique could be useful in the immunization strategy against RSV and PI-3. No plasmid construction or result of vaccination of bovines against these viruses is described in this document.

15 The invention therefore proposes to provide a multivalent vaccine formula which makes it possible to ensure vaccination against a number of pathogenic viruses involved in particular in bovine respiratory pathology and thus to ensure effective vaccination  
20 against this pathology.

Another objective of the invention is to provide such a vaccine formula combining different valencies while exhibiting all the criteria required for mutual compatibility and stability of the  
25 valencies.

Another objective of the invention is to provide such a vaccine formula which makes it possible to combine different valencies in the same vehicle.

30 Another objective of the invention is to provide such a vaccine formula which is easy and inexpensive to use.

Yet another objective of the invention is to provide such a vaccine formula and a method for vaccinating bovines which makes it possible to obtain a  
35 multivalent protection with a high level of efficiency and of long duration, as well as good safety and an absence of residues.

The subject of the present invention is therefore a vaccine formula in particular against bovine

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respiratory pathology, comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one bovine respiratory pathogen valency, these valencies being selected from the group consisting of bovine herpesvirus, bovine respiratory syncytial virus, mucosal disease virus and type 3 parainfluenza virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the bovine herpesvirus, F and G for the bovine respiratory syncytial virus, E2, C + E1 + E2 and E1 + E2 for the mucosal disease virus, and HN and F for the type 3 parainfluenza virus.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus for the pathogen considered, it being possible for the valency to contain, as subvalency, one or more modified or natural genes from one or more strains of the pathogen considered.

Pathogenic agent gene is understood to mean not only the complete gene but also the various nucleotide sequences, including fragments which retain the capacity to induce a protective response. The notion of a gene covers the nucleotide sequences equivalent to those described precisely in the examples, that is to say the sequences which are different but which encode the same protein. It also covers the nucleotide sequences of other strains of the pathogen considered, which provide cross-protection or a protection specific for a strain or for a strain group. It also covers the nucleotide sequences which have been modified in order to facilitate the in vivo expression by the host animal but encoding the same protein.

Preferably, the vaccine formula according to the invention comprises the four valences.

As regards the BHV valency, use is preferably made of the two genes encoding gB and gD, in different plasmids or in one and the same plasmid. Optionally,

For the RSV valency, use is preferably made of the two G and F genes integrated into two different plasmids or into one and the same plasmid. Optionally, but less preferably, the F gene can be used alone.

For the PI-3 valency, use is preferably made of the combination of the two HN and F genes in two different plasmids or in one and the same plasmid. It is also possible to use only the HN gene.

The vaccine formula according to the invention  
20 can be provided in a dose volume of between 0.1 and  
10 ml and in particular between 1 and 5 ml.

25           Use will preferably be made of naked plasmids  
simply placed in the vaccination vehicle which will be  
in general physiological saline (0.9% NaCl), ultrapure  
water, TE buffer and the like. All the polynucleotide  
vaccine forms described in the prior art can of course  
30 be used.

More generally, the promoter may be either of viral origin or of cellular origin. As viral promoter

other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the gene's own promoter.

As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter (Bolmont et al., Journal of Submicroscopic Cytology and Pathology, 1990, 22, 117-122; and Zhenlin et al., Gene, 1989, 78, 243-254), or alternatively the actin promoter.

When several genes are present in the same plasmid, these may be presented in the same transcription unit or in two different units.

The combination of the different vaccine valencies according to the invention may be preferably achieved by mixing the polynucleotide plasmids expressing the antigen(s) of each valency, but it is also possible to envisage causing antigens of several valencies to be expressed by the same plasmid.

The subject of the invention is also monovalent vaccine formulae comprising one or more plasmids encoding one or more genes from one of the viruses selected from the group consisting of BRSV, BVD and PI-3, the genes being those described above. Besides their monovalent character, these formulae may possess the characteristics stated above as regards the choice of the genes, their combinations, the composition of the plasmids, the dose volumes, the doses and the like.

The monovalent vaccine formulae may be used (i) for the preparation of a polyvalent vaccine formula as described above, (ii) individually against the actual pathology, (iii) combined with a vaccine of another type (live or inactivated whole, recombinant, subunit) against another pathology, or (iv) as booster for a vaccine as described below.

The subject of the present invention is in fact also the use of one or more plasmids according to the invention for the manufacture of a vaccine intended to

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vaccinate bovines first vaccinated by means of a first conventional vaccine of the type in the prior art, in particular, selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having, that is to say containing or capable of expressing, the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

Remarkably, the polynucleotide vaccine has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

In general, the first-vaccination vaccines can be selected from commercial vaccines available from various veterinary vaccine producers.

The subject of the invention is also a vaccination kit grouping together a first-vaccination vaccine as described above and a vaccine formula according to the invention for the booster. It also relates to a vaccine formula according to the invention accompanied by a leaflet indicating the use of this formula as a booster for a first vaccination as described above.

The subject of the present invention is also a method for vaccinating bovines against respiratory pathology, comprising the administration of the effective vaccine formula as described above. This vaccination method comprises the administration of one or more doses of the vaccine formula, it being possible for these doses to be administered in succession over a short period of time and/or in succession at widely spaced intervals.

The vaccine formulae according to the invention can be administered, in the context of this method of vaccination, by the different routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

The subject of the invention is also the method of vaccination consisting in making a first vaccination



as described above and a booster with a vaccine formula according to the invention.

In a preferred embodiment of the process according to the invention, there is administered in a first instance, to the animal, an effective dose of the vaccine of the conventional, especially inactivated, live, attenuated or recombinant, type, or alternatively a subunit vaccine, so as to provide a first vaccination, and, within a period preferably of 2 to 6 weeks, the polyvalent or monovalent vaccine according to the invention is administered.

The invention also relates to the method of preparing the vaccine formulae, namely the preparation of the valencies and mixtures thereof, as evident from this description.

The invention will now be described in greater detail with the aid of the embodiments of the invention taken with reference to the accompanying drawings.

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**Sequence listing SEQ ID No.**

- |                 |   |                               |
|-----------------|---|-------------------------------|
| SEQ ID NO. 1    | : | Sequence of the BHV-1 gB gene |
| 35 SEQ ID NO. 2 | : | Oligonucleotide PB234         |
| SEQ ID NO. 3    | : | Oligonucleotide PB235         |
| SEQ ID NO. 4    | : | Oligonucleotide AB162         |
| SEQ ID NO. 5    | : | Oligonucleotide AB163         |

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SEQ ID No. 6 : Oligonucleotide AB026  
SEQ ID No. 7 : Oligonucleotide AB027  
SEQ ID No. 8 : Oligonucleotide AB028  
SEQ ID No. 9 : Oligonucleotide AB029  
5 SEQ ID No. 10 : Oligonucleotide AB110  
SEQ ID No. 11 : Oligonucleotide AB111  
SEQ ID No. 12 : Oligonucleotide AB114  
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SEQ ID No. 14 : Oligonucleotide AB116  
10 SEQ ID No. 15 : Oligonucleotide AB117  
SEQ ID No. 16 : Oligonucleotide AB130  
SEQ ID No. 17 : Oligonucleotide AB131  
SEQ ID No. 18 : Oligonucleotide AB132  
SEQ ID No. 19 : Oligonucleotide AB133

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#### EXAMPLES

##### Example 1: Culture of the viruses

The viruses are cultured on the appropriate  
20 cellular system until a cytopathic effect is obtained.  
The cellular systems to be used for each virus are well  
known to persons skilled in the art. Briefly, the cells  
sensitive to the virus used, which are cultured in  
Eagle's minimum essential medium (MEM medium) or another  
25 appropriate medium, are inoculated with the viral strain  
studied using a multiplicity of infection of 1. The  
infected cells are then incubated at 37°C for the time  
necessary for the appearance of a complete cytopathic  
effect (on average 36 hours).

30

##### Example 2: Extraction of the viral genomic DNAs

After culturing, the supernatant and the lysed  
cells are harvested and the entire viral suspension is  
centrifuged at 1000 g for 10 minutes at +4°C so as to  
35 remove the cellular debris. The viral particles are then  
harvested by ultracentrifugation at 400,000 g for 1 hour  
at +4°C. The pellet is taken up in a minimum volume of  
buffer (10 mM Tris, 1 mM EDTA). This concentrated viral  
suspension is treated with proteinase K (100 µg/ml

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### Example 3: Isolation of the viral genomic RNAs

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## 20

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## 30

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RT-PCR reaction was performed with a pair of specific  
amplimers and taking, as template, the viral genomic RNA  
extracted. The complementary DNA amplified was extracted  
with phenol/chloroform/isoamyl alcohol (25:24:1) before  
5 being digested with restriction enzymes.

**Example 6: plasmid pVR1012**

The plasmid pVR1012 (Figure No. 1) was obtained  
from Vical Inc., San Diego, CA, USA. Its construction  
10 has been described in J. Hartikka et al. (Human Gene  
Therapy, 1996, 7, 1205-1217).

**Example 7: Construction of the plasmid pPB156 (BHV-1 gB  
gene)**

15 The BVH-1 bovine herpesvirus (ST strain)  
genomic DNA (Leung-Tack P. et al. Virology, 1994, 199,  
409-421) was prepared according to the technique  
described in Example 2 and was digested with BamHI.  
After purification, the 18 kbp BamHI-BamHI fragment was  
20 cloned into the vector pBR322, previously digested with  
BamHI, to give the plasmid pIBR-4-BamHI (22 kbp).

The plasmid pIBR-4-BamHI was then digested with  
SalI in order to liberate a 6.6 kbp SalI-SalI fragment  
containing the gene encoding the BHV-1 gB glycoprotein  
25 (Figure No. 2 and SEQ ID No. 1). This fragment was  
cloned into the vector pBR322, previously digested with  
SalI, to give the plasmid pIBR-6,6-SalI (10.9 kbp).

The plasmid pIBR-6,6-SalI was digested with  
NheI and BglII in order to liberate a 2676 bp NheI-  
30 BglII fragment containing the gene encoding the bovine  
herpesvirus (BHV-1) gB glycoprotein (fragment A).

A PCR reaction was carried out with the genomic  
DNA from the bovine herpesvirus (BHV-1) (ST strain) and  
with the following oligonucleotides:

35 PB234 (30 mer) (SEQ ID No. 2)  
5'TTGTCGACATGGCCGCTCGCGGCGGTGCTC 3'  
PB235 (21 mer) (SEQ ID No. 3)  
5'GCAGGGCAGCGGCTAGCGCGG 3'

so as to isolate the 5' part of the gene encoding the

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BHV-1 gB glycoprotein. After purification, the 153 bp PCR product was digested with SalI and NheI in order to isolate a 145 bp SalI-NheI fragment (fragment B).

5 The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pPB156 (7691 bp) (Figure No. 3).

10 **Example 8: Construction of the plasmid pAB087 (BHV-1 gD gene)**

A PCR reaction was carried out with the genomic DNA from the bovine herpesvirus (BHV-1) (ST strain) (P. Leung-Tack et al., Virology, 1994, 199, 409-421), prepared according to the technique described in

15 Example 2, and with the following oligonucleotides:

AB162 (31 mer) (SEQ ID No. 4)

5'AAACTGCAGATGCAAGGGCCGACATTGGCCG 3'

AB163 (30 mer) (SEQ ID No. 5)

5'ATCTTGTACCATATGACCGTGGCGTTG 3'

20 so as to amplify the 5' part of the gene encoding the bovine herpesvirus (BHV-1) gD glycoprotein (GenBank sequence accession No. = L26360) in the form of a 338 bp PCR fragment. After purification, this fragment was digested with PstI and NdeI in order to isolate a

25 317 bp PstI-NdeI fragment (fragment A).

The plasmid pBHV001 (P. Leung-Tack et al., Virology, 1994, 199, 409-421) was digested with NdeI and StyI in order to liberate a 942 bp fragment containing the 3' part of the gene encoding the BHV-1

30 gD glycoprotein (fragment B).

The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with PstI and XbaI, to give the plasmid pAB087 (6134 bp) (Figure No. 4).

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**Example 9: Construction of the plasmid pAB011 (BRSV F gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic

RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch et al., J. Virology, 1991, 181, 118-131), prepared as indicated in Example 3, and with the following oligonucleotides:

5 AB026 (33 mer) (SEQ ID No. 6)  
5'AAAACTGCAGGGATGGCGGCAACAGCCATGAGG 3'  
AB027 (31 mer) (SEQ ID No. 7)  
5'CGCGGATCCTCATTACTAAAGGAAAGATTG 3'

so as to isolate the gene encoding the F fusion glyco-  
10 protein (BRSV F) in the form of a 1734 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 1715 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and  
15 BamHI, to give the plasmid pAB011 (6587 bp) (Figure No. 5).

**Example 10: Construction of the plasmid pAB012 (BRSV G gene)**

20 An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch et al., J. Virology, 1991, 64, 5559-5569) and the following oligonucleotides:

25 AB028 (32 mer) (SEQ ID NO. 8)  
5'AAAACTGCAGATGTCCAACCATACCCATCATC 3'  
AB029 (35 mer) (SEQ ID NO. 9)  
5'CGCGGATCCCTAGATCTGTGTAGTTGATTGATTG 3'

so as to isolate the gene encoding the G protein  
30 (BRSV G) in the form of a 780 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 763 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the  
35 plasmid pAB012 (5634 bp) (Figure No. 6).

**Example 11: Construction of the plasmid pAB058 (BVDV C gene)**

An RT-PCR reaction according to the technique

described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, 74, 1433-1438), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB110 (35 mer) (SEQ ID No. 10)

5'AAAACTGCAGATGTCCGACACAAAAGCAGAAGGGG 3'

AB111 (47 mer) (SEQ ID No. 11)

5'CGCGGATCCTCAATAAAAATCATTCCTGCGACTTGAAACAAAAC 3'

so as to amplify a 342 bp fragment containing the gene encoding the C capsid protein from the BVDV virus. After purification, the RT-PCR product was digested with PstI and BamHI to give a 324 bp PstI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB058 (5183 bp) (Figure No. 7).

#### **Example 12: Construction of the plasmid pAB059 (BVDV E1 "gene")**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, 74, 1433-1438) and with the following oligonucleotides:

AB114 (32 mer) (SEQ ID No. 12)

5'ACGCGTCGACATGAAGAACTAGAGAAAGCCC 3'

AB115 (33 mer) (SEQ ID No. 13)

5'GCGGGATCCTCAGCCGGGTTTGCAAACCTGGGAG 3'

so as to isolate the sequence encoding the BVDV virus E1 protein in the form of a 1381 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1367 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB059 (6236 bp) (Figure



No. 8).

**Example 13: Construction of the plasmid pAB060 (BVDV E2 "gene")**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, 74, 1433-1438) and with the following oligonucleotides:

AB116 (36 mer) (SEQ ID No. 14)  
5'ACGCGTCGACATGACGACTACTGCATTCCTGGTATG 3'  
AB116 (33 mer) (SEQ ID No. 15)  
5'CGCGGATCCTCATTGACGTCCCGAGGTCATTTG 3'

so as to isolate the sequence encoding the BVDV virus E2 protein in the form of a 1252 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1238 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB060 (6107 bp) (Figure No. 9).

**Example 14: Construction of the plasmid pAB071 (BPIV HN gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

AB130 (33 mer) (SEQ ID No. 16)  
5'TTTGTCGACATGGAATATTGGAAACACACAAAC 3'  
AB131 (33 mer) (SEQ ID No. 17)  
5'TTTGGATCCTTAGCTGCAGTTTTTCGGAACCTTC 3'

so as to isolate the gene encoding the BPIV HN glycoprotein (HN gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1737 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1725 bp SalI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6),

previously digested with SalI and BamHI, to give the plasmid pAB071 (6593 bp) (Figure No. 10).

**Example 15: Construction of the plasmid pAB072 (BPIV F gene)**

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

10 AB132 (30 mer) (SEQ ID No. 18)

5' TTTGTCGACATGATCATCACAACACAATC 3'

AB133 (30 mer) (SEQ ID No. 19)

5' TTTGGATCCTCATTGTCTACTTGTAGTAC 3'

15 so as to isolate the gene encoding the BPIV F glycoprotein (F gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1641 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1629 bp SalI-BamHI fragment. This fragment  
20 was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB072 (6497 bp) (Figure No. 11).

**Example 16: Preparation and purification of the plasmids**

For the preparation of the plasmids intended for the vaccination of animals, any technique may be used which makes it possible to obtain a suspension of purified plasmids predominantly in a supercoiled form.

30 These techniques are well known to persons skilled in the art. There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in  
35 J. Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Reference may also be made to Patent Applications PCT WO 95/21250 and PCT WO 96/02658, which describe methods for producing, on an

industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines (see Example 17), the purified plasmids are resuspended so as to obtain solutions at a high concentration (> 2 mg/ml) which are compatible with storage. To do this the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

10 **Example 17: Manufacture of the associated vaccines**

The various plasmids necessary for the manufacture of an associated vaccine are mixed starting with their concentrated solutions (Example 16). The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

Specific formulations such as liposomes or cationic lipids, may also be used for the manufacture of the vaccines.

**Example 18: Vaccination of bovines**

The bovines are vaccinated with doses of 100 µg, 250 µg or 500 µg per plasmid. The injections are performed with a needle by the intramuscular route either at the level of the *gluteus* muscle, or at the level of the neck muscles. The vaccinal doses are administered in volumes of between 1 and 5 ml.

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